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Diminished *Plasmodium falciparum* sensitivity to quinine exposure in vitro and in a sequential multi-drug regimen
A preliminary investigation in Guyana, South America

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Summary
Objectives: This preliminary study sought to investigate the response of uncomplicated falciparum infections to semi-supervised drug administration with quinine and two adjunctive schizontocidal drugs in infected patients in Guyana. Quinine and chloroquine cross-sensitivity was also assessed in vitro.
Methods: Patients were treated with quinine 10 mg/kg for 7 days followed by sulfadoxine/pyrimethamine 25 mg/kg single dose (in children) or doxycycline 100 mg daily for 7 days (in adults). Independently, falciparum-infected blood-medium mixtures were cultured in standardized pre-dosed quinine and chloroquine test plates, according to the protocol of the World Health Organization Mark III in vitro test system, for analysis.
Results: The quinine/doxycycline regimen ($N = 12$) produced 100% clinical cure (12/12) at day 14 and 100% parasitological cure (11/11) at day 28. However, with the quinine/sulfadoxine/pyrimethamine scheme, 1/12 therapeutic failure (on day 14) and 2/9 parasitological failures (on day 28) were observed. In vitro, parasite development beyond the cut-off concentrations and high IC_{50} values (geometric mean IC_{50} quinine 504.65 nM and IC_{50} chloroquine 506.69 nM), confirmed diminished *Plasmodium falciparum* sensitivity to both drugs.
Conclusion: These findings suggest *P. falciparum* resistance to both quinine and chloroquine, and support either the use of antibiotics as adjuncts to quinine therapy or drugs with alternate pharmacodynamics as first-line therapy.

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Introduction

Sequential drug administration has been successfully employed to prolong drug activity in managing infectious diseases.[1] As such, in response to the consistently observed failure of chloroquine in 1975 and subsequently, in 1978, of sulfadoxine/pyrimethamine monotherapy to cure *Plasmodium falciparum* infections in Guyana, a protocol for the sequential treatment of uncomplicated infections with quinine sulfate and sulfadoxine/pyrimethamine was employed between 1984 and 2001.[2,3]

Guyana is a South American country (86 000 sq. miles) with a population of approximately 721 000 persons.[4] While most of the residents live on a narrow coastal strip that is largely devoid of active malaria transmission (Figure 1), malaria remains endemic in the interior geopolitical regions, i.e., Regions 1, 7, 8, and 9. In this primarily Amazonian tropical rainforest district, the sylvatic *Anopheles darlingi* is considered the primary vector for malaria transmission.[5,6] Here also, the resident population is small and scattered, but there is a sizeable itinerant group of miners and loggers that moves not only throughout the interior and coastal areas of Guyana, but also into the neighboring countries of Brazil, Venezuela, and Suriname. The available healthcare facilities are basic with no regular electricity supply, and care is provided primarily by community health workers and medical extension officers (Medexes) at health centers or smaller health posts. When possible or necessary, patients travel to the capital city Georgetown for treatment.

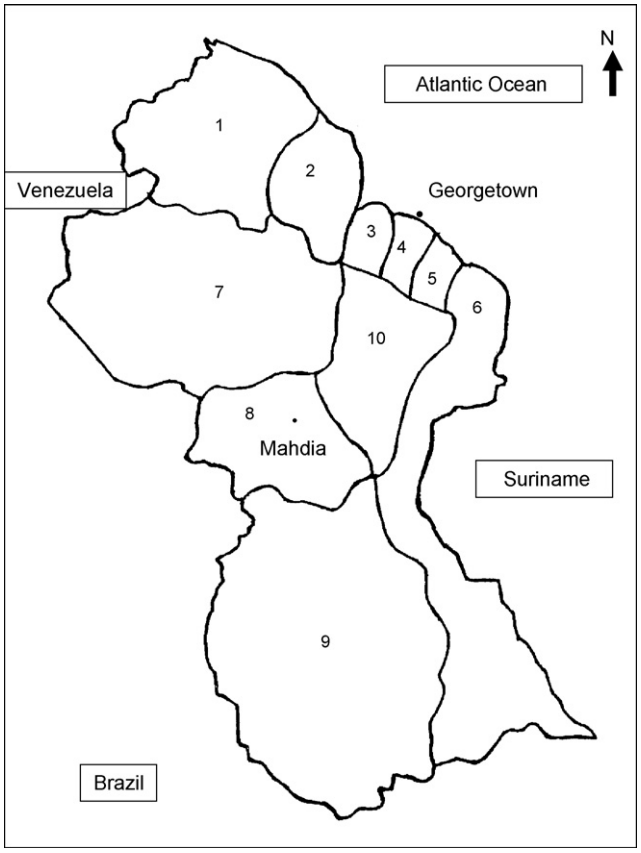

Figure 1 Map of Guyana showing the 10 administrative geopolitical regions and study sites.

Recognizing the persistent problem of patient non-compliance since the introduction of the sequential quinine combination regimens in Guyana,[7,8] and the consequent possibility of natural selection of drug-resistant parasites after years of variable drug pressure, this study was conducted in an endemic community to gather preliminary data on the in situ effectiveness of two treatment regimens utilizing quinine with either sulfadoxine/pyrimethamine or doxycycline. To identify trends in parasite responsiveness to quinine and chloroquine, the in vitro responses of *P. falciparum* to both drugs were determined using isolates obtained from a second group of patients who had been infected in the same endemic areas, but who had traveled to the city for treatment.

Methods

The protocols were approved by the Ethics Committee of the Faculty of Medical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago and the Institutional Review Board of the Ministry of Health, Guyana. The studies were conducted between May and October 2001. The clinical study was executed at the Mahdia District Hospital (Region 8) in the interior of Guyana, while the in vitro studies were done in the coastal capital city Georgetown at the Vector Control Services (VCS) referral clinic of the Georgetown Hospital and at the Eureka Laboratory. All patients or a parent/guardian signed informed consent prior to participation.

Clinical study

Subjects

Owing to the exigencies associated with conducting clinical studies in the malaria-endemic communities of Guyana, and in the interests of time and cost, a convenient sample was used for this preliminary study. Consenting ambulatory residents (>6 months age) living in Mahdia for at least 6 months, with a minimum diagnosis of uncomplicated asexual stage *P. falciparum* infection (parasite count $\geq$500 parasites/μl blood following an initial relative parasite count by the VCS of at least +F or 1−10 falciparum parasites per 100 thick film fields), and a recent history of fever, were recruited consecutively into the study. Exclusion criteria[9] included pregnancy (self-reported last menstrual period), breast feeding, symptoms of severe malaria, reported current or recent use (in the past 2−4 weeks) of quinine, chloroquine, doxycycline, mefloquine, or halofantrine, a positive Dill and Glazko[10] urine test, and current or recent use of herbal extracts/infusions of local herbs used to treat symptoms of malaria. Demographic data and a recent history of malaria were collected, and after a clinical examination by the doctor or trained paramedical staff (Medex), patients were asked to remain in the study area for 28 days of follow-up.

Management

Patients were placed into one of two treatment groups: adults (age $\geq$14 years) and children (age 6 months to <14 years). Based upon the national treatment schedules[3] in use at that time, patients received from the research team or a

family member, quinine sulfate tablets (10 mg salt/kg every 8 hours; Novopharm Ltd, Toronto, Canada) for 7 days. For adults, this was followed on day 8 by doxycycline tablets 100 mg daily (International Dispensary Association, Holland) for seven additional days, while children, received a single sulfadoxine/pyrimethamine tablet (sulfadoxine 25 mg/kg; International Dispensary Association, Holland) on day 8. A single dose of primaquine 0.75 mg/kg (up to 45 mg in adults; International Dispensary Association, Holland) was given along with the first quinine dose in both groups of patients as a gametocytocidal agent. Febrile patients (>37.5 °C at enrolment) were given acetaminophen (International Dispensary Association, Holland) on admission into the study, and for subsequent self-administration if necessary. Temperature was recorded on days 1–8, 14, 21, and 28, and Giemsa-stained thick blood films were evaluated 24 and 48 hours after enrollment into the study,[11] and on days 4, 6, 7, 14, 21, and 28. Reports of likely adverse effects were taken at each visit, and patients were advised to return to the research team if they experienced any illnesses between visits. Therapeutic response to the sequential regimen was assessed on day 14 according to World Health Organization (WHO) criteria,[9] while parasitologic response was determined on day 28. Since all patients were remaining in an area of ongoing transmission, pyrethroid-treated bed-nets, mosquito coils, and skin repellent were supplied with advice on their appropriate usage, to reduce the likelihood of reinfection during the 28-day period.

Ethical considerations for withdrawal

Patients were considered for withdrawal from the study if the initial diagnosis could not be re-confirmed by a senior microscopist, parasitemia or symptoms worsened between days 1 and 6, or adverse effects of drugs were intolerable. In all cases, the patients would be referred to the VCS for appropriate clinical assessment and completion of therapy.[12]

In vitro study

Subjects

In Georgetown, patients were selected for the in vitro study using the same procedure and exclusion criteria applied for obtaining the sample for the clinical study. Patient interviews provided information on their demographic profiles, recent history of malaria, and use of antimalarial preparations.

Culturing of parasites

The protocol of the WHO Mark III in vitro test system[13] was employed with standardized pre-dosed chloroquine and quinine test plates prepared by the Vector Control Research Unit of the Universiti Sains Malaysia, Penang, Malaysia by arrangement with the WHO Western Pacific Regional Office. All tests were run in triplicate. After incubation, thick films of the red blood cell precipitate were stained with 2% Giemsa solution and the numbers of schizonts with three or more merozoites per 200 asexual parasites were counted.

The recommended cut-off points for parasite resistance in the WHO Mark III system,[13] are growth of schizonts with three or more nuclei at a concentration of 5.12 μmol/l (quinine) and 12.8 μmol/l (chloroquine).

Data analysis

Student's *t*-test was used to compare the mean parasitemia at enrolment of patients in both treatment arms of the clinical study. A 95% level of significance was applied in all tests. Confidence intervals for evaluable treatment outcomes were computed using an online calculator (http://www.dimensionresearch.com/resources/calculators/conf_prop.html). Inhibitory concentrations for each drug used in the in vitro test were determined by non-linear regression analysis using the HN-NonLin V1.1 computer program developed by Dr Harald Noedl for the analysis of in vitro drug sensitivity data (http://www.who.int/malaria/rbm/Attachment/20041108/NonLin_V1.1.xls). The data were graphed following adaptation to a log-probit model. Parasite activity to quinine and chloroquine as measured by IC_{50} and IC_{90} values was compared by using Spearman's coefficient at the two-tailed level of significance.

Results

Clinical findings

Although 32 patients were enrolled initially into the clinical study, five patients were withdrawn for reasons unrelated to the treatment protocol and an additional three were lost to follow-up early in their treatment due to work-related migration. Eventually 24 patients including 12 adults (median age 23 years) and 12 children (median age 6.5 years) were successfully followed up for clinical resolution (14 days), and 20 patients (11 adults and nine children) for parasitologic resolution (28 days). There was no significant difference in the mean parasitemia ($p = 0.24$) between the two treatment groups at recruitment.

Table 1 summarizes the results of the two treatment arms. Among children (QPS), one patient had both clinical and parasitologic failure (day 11 of treatment), while in another patient, only parasitologic failure was reported, as parasitemia recurred on day 17 of treatment. Two intercurrent *Plasmodium vivax* infections (which were censored) were also diagnosed in this group on days 14 and 23. All of these infections were symptomatic. The recurrent *P. falciparum* and intercurrent *P. vivax* infections were referred to the VCS for subsequent management. All adults (QPD) who completed follow-up showed an adequate clinical response at day 14 and parasitologic sensitivity up to day 28. Ringing in the ears or deafness was the most commonly reported adverse effect (100% of persons reporting) in the first week of therapy.

In vitro results

Of 30 persons interviewed, 23 patients infected in the endemic area satisfied the inclusion criteria. Median age was 24 years and the geometric mean parasitemia at recruitment was 7419.2 parasites/μl blood. The %SMI (schizont maturation inhibition) values suggested reduced sensitivity to quinine in 6/14 samples and to chloroquine in 11/14 samples, although three of the latter were considered only borderline resistant. Twelve samples (52%) were successfully cultured for both quinine and chloroquine, and the assessment of their quinine/chloroquine cross-sensitivities by correlation analysis found a weak and insignificant relationship at the IC_{50}

Table 1 Treatment outcomes (cure/failure) for both the clinical and parasitological end points measured

Treatment		Day 14 (clinical)	Day 28 (parasitological)
QPD (12) Adults	*N*	12	11
	Cure (95% CI)	12	11
		100% (75—100)	100% (73.5—100)
	Failure (95% CI)	0	0
		0% (0.0—24.7)	0% (0.0—26.5)
QPS (12) Children	*N*	12	9
	Cure (95% CI)	11	7
		91.7% (63.9—98.1)	77.8% (44.4—93.3)
	Failure (95% CI)	1 (day 11)	2 (day 11, day 17)
		8.3% (0.2—36.0)	22.2% (6.7—55.6)

QPD, quinine/primaquine/doxycycline; QPS, quinine/primaquine/sulfadoxine/pyrimethamine.

level that strengthened at the IC_{90} level (Table 2). The log-probit graphs for quinine and chloroquine (Figure 2) in comparison seemed to suggest a trend to correlation between the responses to the two drugs at the lower drug concentrations, with quinine possibly displaying greater potency as the slope of its regression line appeared steeper.

Discussion

Decisions to modify national treatment protocols in Guyana have for many years been based upon clinical observations in a range of patients. This has been as a consequence of the unavailability of scientific data, as clinical studies following either WHO or other established protocols were not routinely conducted by the National Malaria Control Programme. While clinical studies provide an opportunity to discern the degree of therapeutic failure in the context in which it is likely to be manifested in an endemic community, in vitro tests preferentially highlight specific drug-sensitivities of parasites, and expose subtle response trends.

In Guyana, chloroquine had been used successfully to manage falciparum infections for many years, but was discontinued in the light of clinical observations and reported spreading drug resistance in neighboring countries (Brazil,[14] Suriname,[15] and Venezuela[16]). In 2002, the clinical responses of a group of *P. vivax* and *P. falciparum* infected Guyanese patients given observed treatment with chloroquine was described.[8] Although providing good symptomatic relief for the patients with *P. falciparum*, chloroquine at that time nevertheless produced 48% therapeutic failure by day 14 and 55% parasitologic failure by day 28. Our in vitro findings reported here, although derived from a small sample, highlight a similar heterogeneity of the parasite population, featuring a mix of chloroquine sensitive and resistant parasites with a greater tendency towards drug resistance.

As a result of the prevalence of chloroquine resistance in this geographic region, the possibility of a compromised parasite response to the analog quinine, as observed in other countries,[17—19] was likely. In this context, the D1246Y, S1034C, and N1042D *pfmdr1* mutations, which are associated with quinine resistance in Brazil,[17] had also been reported in molecular studies on chloroquine-resistant isolates from Guyana[20] hinting at a relationship between parasite sensitivity to these drugs. As such, activity correlation analyses were undertaken to investigate this further.[21] This preliminary study however, likely due to the small sample size, did not find good evidence of correlation between chloroquine and quinine activity in vitro at the IC_{50} level. Further the stronger yet negative activity relationship at the IC_{90} level suggests diverging mechanisms of action at higher drug concentrations, and deserves to be verified in larger studies.

While the apparently waning parasite susceptibility to quinine clinically, and the trends of the log-concentration

Table 2 In vitro indices of parasite responses to the two drugs chloroquine and quinine showing the mean 50% and 90% (IC_{50} and IC_{90}) inhibitory concentrations for the samples and a correlation analysis of parasite cross-sensitivities to the two drugs

	IC_{50} (nmol/l)[a]	IC_{90} (nmol/l)[a]
Quinine (*N* = 14)	504.651	1526.904
Chloroquine (*N* = 14)	506.6914	1579.172
N (pairs Cq/Q)	12	12
R (Spearman's)	0.190	−0.529
p	0.554	0.077
Significance	NS ($p > 0.05$)	NS ($p > 0.05$)

[a] Geometric means of the IC_{50} and IC_{90} values. Cq, chloroquine; Q, quinine.

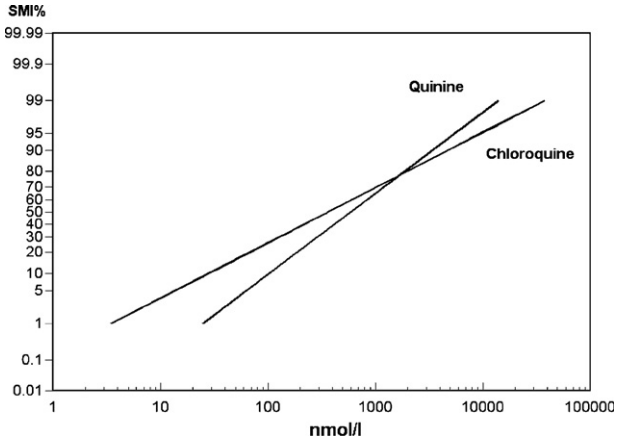

Figure 2 Log-probit regression curves comparing in vitro parasite inhibition by quinine and chloroquine (SMI = schizont maturation inhibition).

probit regression lines for parasite responses to quinine and chloroquine described here, evince similarity in the mechanisms of resistance to the two drugs at low levels of resistance, the presence of other influences such as independent mechanisms of action for quinine at higher drug levels, cannot be ignored. These observations allude to a likely role for factors apart from the widely speculated non-compliance[7,8] in the observed failure of the quinine/sulfadoxine/pyrimethamine regimen to produce a clinical and parasitologic cure consistently. The current findings also emphasize the need to comprehensively study the drug sensitivities of the *P. falciparum* parasite population in Guyana, in an effort to support the most pharmacoeconomic and rational choice of treatment regimens in the cash-strapped public sector.

A major limitation of the current clinical study was the inability to use cytogenetic studies to differentiate between recrudescence and reinfection in the clinical cases of recurrent parasitemia. However if self-reported compliance with the treatment regimen is accepted, the recurrence of symptoms and parasitemia within 11 days in one case while using more than one intervention against exposure, mitigates the likelihood of reinfection. Still the small sample sizes limit clear conclusion of the superiority of one drug or drug regimen over the other, again underscoring the importance of extending these studies to confirm the findings.

In conclusion, these preliminary findings indicate declining sensitivity of *P. falciparum* to quinine in Guyana. As the use of other quinoline-related drugs (mefloquine and halofantrine) spreads and new schizontocidals (artemesinin derivatives) are introduced as first-line drugs for falciparum malaria in Guyana, periodic larger clinical and in vitro studies will be needed to inform the National Malaria Control Programme on the dynamics of parasite cross-sensitivities within the local population. Finally, since quinine remains the national drug of choice for severe malaria and for the treatment of both children under 5 years and pregnant women in their first trimester however, conservation of its usefulness must also be addressed.

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